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COMMUNICATION

Peptide functionalised discotic amphiphiles and their self-assembly into supramolecular nanofibres†

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The multicomponent co-assembly of discotic amphiphiles provides a modular and versatile approach to prepare RGDS- and PHSRN-peptide functionalised supramolecular nanofibres, bearing pendant paramagnetic Gd(III)-chelates.

Supramolecular engineering of soft-condensed matter has allowed chemists to develop robust and versatile methodologies for the preparation of solution based macromolecular architectures, gels and bulk materials.[1] Particularly for biomedical applications,[2] non-covalent architectures in aqueous environments are indispensable, relying on self-assembled linear amphiphiles, such as phospholipids,[3] block copolymers[4] or peptide amphiphiles.[5] Stupp and coworkers have shown that the latter are extremely appealing owing to their modular synthetic approach: multifunctional and multivalent non-covalent nanofibres can be prepared conveniently without the need for repeated syntheses, obtaining scaffolds that are dynamic and adaptable, yet inherently degradable into their small molecular weight constituent subunits.[2b,5b,6]

Recently, we[1a,7] and others[1h,8] have reported a new class of C_3-symmetrical discotic amphiphiles that are designed to polymerise into columnar aggregates, *via* a combination of hydrogen bonding, π–π interactions and solvophobic effects. In order to demonstrate the versatility of the benzene-1,3,5-tricarboxamide (BTA) based supramolecular polymers, we hereby report the preparation of multifunctional supramolecular nanorods, *via* the multicomponent co-assembly of two different peptide functionalised discotics (RGDS and PHSRN), and fluorophore [carboxyfluorescein (CF)] pendant discotics, with MRI (Gd^{III}–DOTA) labelled amphiphiles (Fig. 1). Nanorods, functionalised with synergistic peptide units as well as MRI labels could be used as targeted contrast agents in molecular imaging applications. These nanoscopic, multifunctional and multivalent constructs would thereby allow for high contrast agent efficacies in combination with accumulation in an area of interest. We show that stacking of peptide bearing discotic amphiphiles into one-dimensional nanorods can lead to reinforced aggregation, bundling and hence the formation of nanofibres. Furthermore, by mixing in Gd^{III}–DOTA labelled discotic amphiphiles that act as diluting co-monomers, secondary interactions are avoided and well-defined nanorods of 50 nm in length are obtained. In line with our previous investigations, we will highlight the need to correlate the self-assembly pathways of the ordered supramolecular polymers with morphological characterisations.[1a,7,9]

The synthetic route was designed around a common C_3-symmetrical intermediate that was prepared in a convergent approach (Scheme 1). Thereafter Gd(III)–DOTA and CF labels could be attached *via* their activated ester moieties (Scheme 2).[1a] In comparison, the preparation of peptide functionalised discotics was thought to be more challenging. Since the purification of multivalent supramolecular monomers that are highly amphiphilic is not straightforward, our approach involved an orthogonal and highly efficient ligation step that could be performed under mild conditions and at the same time, avoid protecting group strategies. For this reason, the

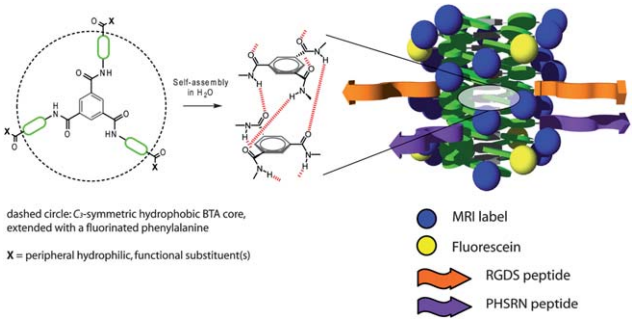

Fig. 1 Schematic representation of the self-assembly of discotic amphiphiles: the hydrophobic BTA core directs the self-assembly into a helical architecture whilst the peripheral hydrophilic functional groups introduce an amphiphilic character.

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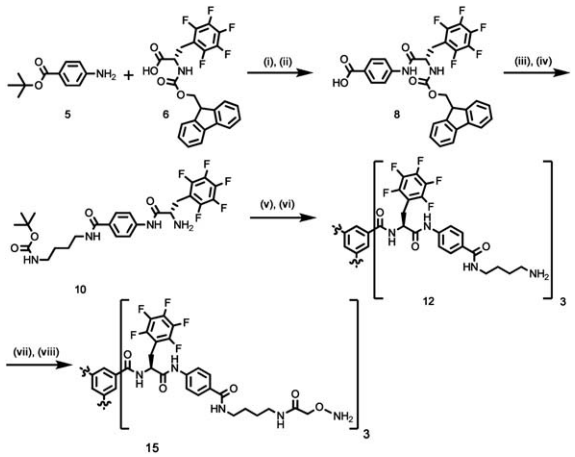


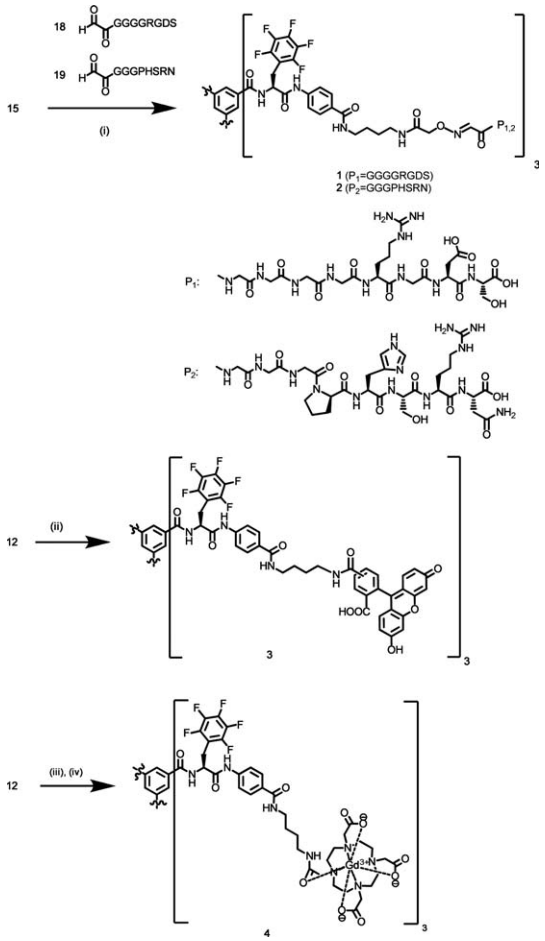
Scheme 1 Synthesis of the nucleophilic, hydrophobic cores of the discotic synthetic precursors **12** and **15**: (i) HATU, DiPEA, DMF, 25 h, 90%; (ii) TFA–DCM (1 : 1), 5.5 h, 98%; (iii) PyBOP, DiPEA, THF, 12 h, 80%; (iv) piperidine, CH_3CN, 1.5 h, 53%; (v) THF–DMF (1 : 9), DiPEA, 36 h, 72%; (vi) TFA–DCM (1 : 1), 2.5 h, 72%; (vii) **14**, DiPEA, DMF, 2 h, 49%; (viii) TFA–DCM (1 : 1), 0 °C, 1 h, quantitative.§

use of oxime ligation seemed to meet all these requirements (Scheme 2).[10] The target peptides were the well-known integrin binding ligands Gly-Arg-Gly-Asp-Ser (GRGDS) and Pro-His-Ser-Arg-Asn (PHSRN).[11] A short, flexible glycine spacer (GGG) and a serine (S) were introduced to the *N*-terminus of the peptides and synthesized by solid phase peptide synthesis, using standard Fmoc-based protocols. Without purification of the peptides, the *N*-terminal serine of both peptides was selectively oxidized using periodate ($NaIO_4$) at neutral pH.[10b]

Aggregation and therefore steric hindrance are the most likely explanations for the unexpectedly long reaction times to achieve full conversion for the oxime ligations. However, the conversions and isolated yields for all the intermediate synthetic steps were very high and highly pure products were obtained (Fig. 2). Only in the final purification step *via* preparative liquid chromatography, material was lost due to the tendency for the structures to aggregate during chromatography.

In the simplest case of a single peptide discotic aggregate, solutions of **1** or **2** in dilute buffered conditions give rise to strong Cotton effects indicating the formation of helical supramolecular polymers (Fig. 3A). However, when monitoring the negative CD band at 280 nm—which is characteristic for ordered aromatic chromophores of the hydrophobic wedge—at different temperatures, we observed extraordinarily high stability of these aggregates (Fig. 3B). At 1×10^{-5} M of **1**, the CD effect only dropped by about 10% after heating the solutions to 368 K. Only upon addition of 5 vol% DMSO, a strong hydrogen bond acceptor, and further diluting the system to 2.5×10^{-6} M, were the non-covalent polymers fully disassembled. Peptide discotic **2** showed very similar aggregation behaviour. This is a rare case in which we have observed such stable ordered supramolecular polymers in aqueous environments (Fig. 4).

By monitoring the temperature dependence of the CD band at 280 nm from a molecularly dissolved (*i.e.* 368 K) to fully aggregated state (*i.e.* 283 K), we studied the assembly process in more detail. The cooling curves or growth profiles observed were reminiscent of a nucleation–elongation type mechanism at high temperatures. However at about 320 K for **1** or already at 340 K in the case of **2**, the



Scheme 2 Synthesis of the peptide functionalised discotic amphiphiles **1** and **2**, carboxyfluorescein (CF, mixture of isomers) and Gd(III)–DOTA pendant discotics **3** and **4**: (i) anilinium acetate buffer (0.1 M, pH 4.5), 5 days, 10%; (ii) CF–NHS, DiPEA, DMF, 25 h, 76%; (iii) DOTA–NHS, DiPEA, DMF, 12 h, quantitative; (iv) $Gd(OAc)_3 \cdot H_2O$, ultra pure water, pyridine, 50%.§

curves started to level off or even increase. These additional transitions in the cooling curves strongly indicate a secondary process, most likely arising from interactions of the peptide side arms within one stack. This would also explain the extraordinary high stability of the rod-like structures compared to the amphiphiles reported previously.[1a] To find further evidence for this hypothesis we performed cryo-TEM, in the same buffered conditions. Very long rods in the micrometre range were observed which exhibited a high tendency to

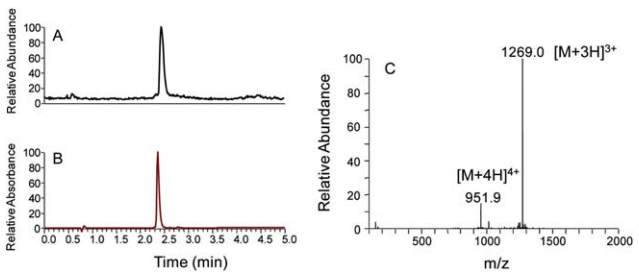


Fig. 2 LC-MS analysis of purified peptide discotic **1**: (A) TIC trace, (B) PDA trace, and (C) ESI-MS.

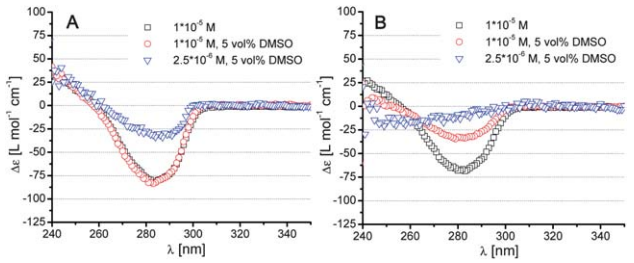


Fig. 3 CD spectra of **1** at different concentrations in citrate buffer (100 mM, pH 6), and increasing amounts of DMSO: spectra at (A) 283 K and (B) 368 K.

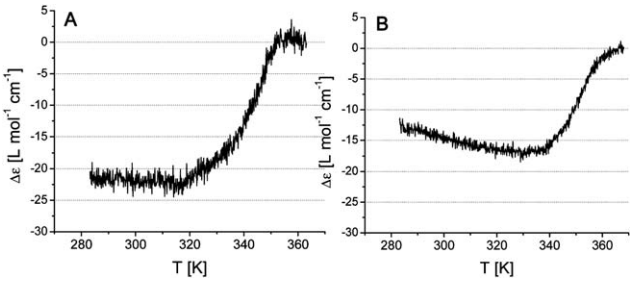


Fig. 4 Temperature dependent CD spectra of (A) **1** and (B) **2** (2.5×10^{-6} M), in citrate buffer (100 mM, pH 6, 5 vol% DMSO), monitored at $\lambda =$ 280 nm.

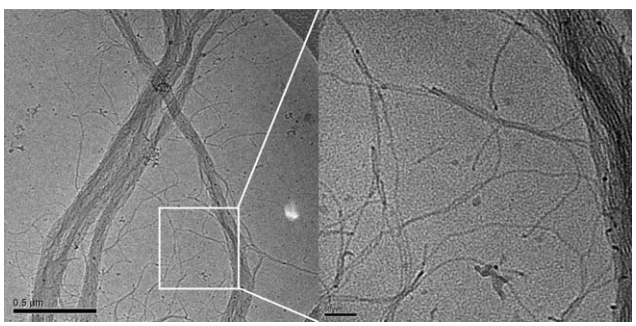


Fig. 5 Cryo-TEM of aggregates based on peptide discotic **1** at 1 mg ml^{-1} in citrate buffer (100 mM, pH 6); scale bars represent 0.5 μm (left) and 50 nm (right).

bundle up into fibres (Fig. 5). This further confirms secondary interactions within 1D aggregates.

In the past we have shown the cooperative self-assembly of Gd(III)–DOTA discotics **4** into rods of 50–75 nm at millimolar concentrations.[1a] By combining peptide discotics **1** and **2** with **4** we were aiming at targeted MRI labels. However, before using the co-aggregates for this application insights into the processes of co-assembly are required, since small changes in their composition can alter the behaviour drastically. At an overall concentration of 0.5 mM (1 mg ml^{-1}), a mixture of **4** were co-assembled with RGDS- and PHSRN-labelled discotics **1** and **2** in a ratio of 12 : 1 : 1, respectively. After addition of a buffer to the solid compounds and sonication yielded clear solutions, which were previously not observed for the peptide discotics themselves due to scattering of the clustered fibres.

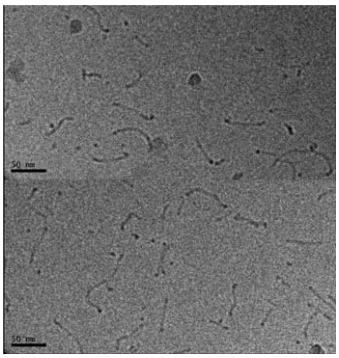


Fig. 6 Cryo-TEM of aggregates based on peptide discotics **1**, **2** and Gd (III) labelled discotic **4** (1 mg ml^{-1}, molar ratio of 1 : 1 : 12) in citrate buffer (100 mM, pH 6); scale bars represent 50 nm.

Cryo-TEM images clearly show that single nanorods were produced of at least 50 nm in length (Fig. 6), which corresponds to a degree of polymerisation of about 150, assuming a disc–disc spacing of 0.35 nm,[12] and molecular weights in the order of 500 kDa. This implies that these rods are functionalised with around 66 peptide units of RGDS and PHSRN (1 : 1) and provide a very promising platform for potential multivalent and synergistic binding studies.

For further evidence of the successful co-assembly of peptide functionalised and diluting discotics, a series of CD experiments were performed. In contrast to the single monomer **1** and **2** based aggregates, the supramolecular copolymerisation yielded CD effects similar to the one-dimensional rods based on discotic **4** alone.[1a] Importantly the cooling curves show a self-assembly process lacking any secondary interactions. The shape is consistent with a nucleation–elongation type mechanism, typical for a cooperative polymerisation and follows the growth profile reported previously for the homopolymerisation of **4**. Similar growth profiles were observed for different ratios of peptide to Gd(III) labelled discotics, ranging from 1 : 6 to 1 : 3 (Fig. 7).

To follow the self-assembly process by temperature dependent photoluminescence (PL), CF bearing discotic **3** was introduced to the heterologous supramolecular aggregates. Critical temperatures in PL emission intensities overlap well with those observed in CD cooling curves (Fig. 7). The aggregation induced increase in fluorescence intensity is significantly higher than the temperature dependent emission quantum yield of CF in the same buffered conditions (see ESI†). A series of control experiments at different ratios of CF labelled discotics to diluting discotics confirm that at least a ratio of 1 : 10 is necessary to observe profiles as depicted in Fig. 7D. A lower ratio results in quenching between laterally stacked CF units that are in close proximity. The incorporation of the labelled discotics into macromolecular stacks was confirmed by fluorescence anisotropy experiments (see ESI†).

In conclusion, we have shown that multicomponent self-assembly is a versatile methodology to prepare nanosized soft materials with peripheral functional groups, in this case, pendant peptides, Gd(III) chelates and fluorescein groups. Stacking of the peptide discotic amphiphiles into one-dimensional arrays leads to reinforced aggregation induced by weak secondary interactions between the peripheral groups. Modular self-assembly, using diluting discotic amphiphiles, prevents these secondary interactions due to the spatial separation of peptide bearing discotics. Temperature dependent CD and PL were sensitive techniques to investigate the self-assembly

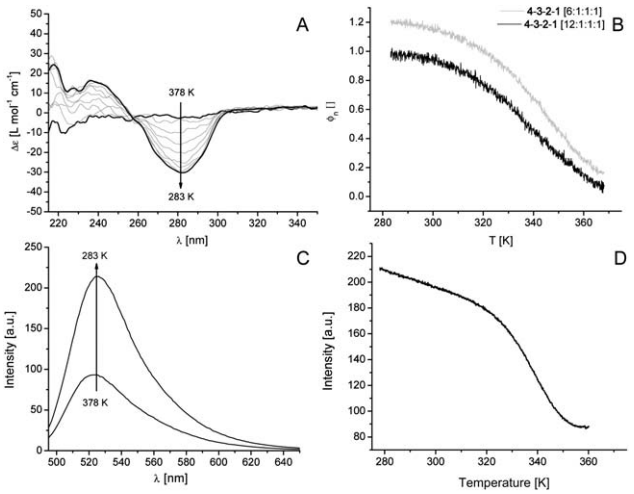


Fig. 7 (A) Temperature dependent CD spectra for **4–3–2–1** [12 : 1 : 1 : 1] in PBS buffer [10 mM, pH 7.4]; (B) normalised CD cooling curves at ratios of 12 : 1 : 1 : 1 and 6 : 1 : 1 : 1 [degree of aggregation ϕ *vs.* temperature: 0 = the molecular dissolved state, 1 = fully polymerised system, grey curve is offset for clarity], (C) temperature dependent PL spectra (λ_{ex} = 485 nm) for **4–3–2–1** [12 : 1 : 1 : 1] and (D) cooling curve measured at λ_{em} = 525 nm; in all cases the total discotic concentration was 5×10^{-6} M.

process and to probe changes in solution morphologies. These studies highlight the importance of understanding the self-assembly pathways for multicomponent supramolecular polymers. This is crucial for determining ideal mixing ratios of the building blocks and optimising the distribution of functional groups for molecular imaging applications.

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Notes and references

§ The specified reactions were performed at room temperature and in dry solvents, under Argon.

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